UNITED STATES PATENT AND TRADEMARK OFFICE
DOCUMENT CLASSIFICATION BARCODE SHEET

CATEGORY:

CLEARED

ADDRESS
CONTACT IF FOUND:

520 Rec'd PCT/PTO 18 APR 2000

FORM PTO-1390 U.S.DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (REV 5-93)

ATTORNEY'S DOCKET NUMBER
0 050000/REF/BJD

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371

U S APPLICATION NO (IF KNOWN, SEE 37 C F R. § 1.5)
TO BE ASSIGNED 09/529659

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP98/06546	October 15, 1998	October 18, 1997

TITLE OF INVENTION
Tumour Vaccine

APPLICANT(S) FOR DO/EO/US
Ernst WAGNER, Ralf KIRCHEIS, Daan J.A. CROMMELIN, Maaike van SLOOTEN, and Gert STORM

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. § 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☐ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ☒ A translation of the International Application into English (35 U.S.C. § 371(c)(2)).

7. ☐ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. § 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 372(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. § 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. § 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. § 3.28 and 3.31 is included.

13. ☐ A FIRST preliminary amendment.

14. ☐ A SECOND or SUBSEQUENT preliminary amendment.

15. ☐ A change of power of attorney and/or address letter.

16. ☐ Other items or information:

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.50)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/529,659	PCT/EP98/06546	0652.2050000/REF/BJD

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO $840.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) . $670.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $690.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $970.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... $ 96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	- 20 =		X $18.00	$	
Independent Claims	- 3 =		X $78.00	$	
Multiple dependent claim(s) (if applicable)			+ $260.00	$	
TOTAL OF ABOVE CALCULATIONS			=	$	
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity statement must be filed. (Note 37 CFR 1.9, 1.27, 1.28)				$	
SUBTOTAL =				$	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover (37 CFR 3.28, 3.31). $40.00 per property +				$ 40.00	
TOTAL FEES ENCLOSED =				$ 40.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ 40.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0036. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit Under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
1100 New York Avenue, NW, Suite 600
Washington, D.C. 20005-3934

Raz Fleshner — Signature
June 22, 2000 — Date
Raz E. Fleshner — Type Name
34,331 — Registration Number

416 Rec'd PCT/PTO 18 APR 2000

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
TO BE ASSIGNED 09/529659	PCT/EP98/06546	0652.2050000/REF/BJD

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO $840.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) . $670.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $690.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $970.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... $ 96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

Claims	Number Filed		Number Extra	Rate		
Total Claims	17	- 20 =	0	X $18.00	$ 00.00	
Independent Claims	1	- 3 =	0	X $78.00	$ 00.00	
Multiple dependent claim(s) (if applicable)				+ $260.00	$ 260.00	
TOTAL OF ABOVE CALCULATIONS =					$ 1230.00	
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity statement must be filed. (Note 37 CFR 1.9, 1.27, 1.28)					$	
SUBTOTAL =					$ 1230.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +					$	
TOTAL NATIONAL FEE =					$ 1230.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover (37 CFR 3.28, 3.31). $40.00 per property +					$	
TOTAL FEES ENCLOSED =					$ 1230.00	
					Amount to be: refunded	$
					charged	$

a. ☒ A check in the amount of $ 1230.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0036. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit Under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
1100 New York Avenue, NW, Suite 600
Washington, D.C. 20005-3934

Brian J. Del Buono
Signature

April 18, 2000
Date

Brian J. Del Buono
Type Name

42,473
Registration Number

Tumour vaccines

The invention relates to the field of the immunotherapy of tumoral diseases. The majority of malignant tumours successfully escape control by the immune system, even though a number of them express tumour-associated antigens (van der Bruggen, 1991; Brichard, 1993; Gaughler, 1994; Coulie, 1994). It has been found that, in addition to the presentation of the appropriate antigens, certain adjuvants are also needed in order to achieve effective stimulation of the immune system (Zier, 1995).

Early studies were aimed at stimulating the immune system by vaccinating with inactivated tumour cells in conjunction with non-specific immunostimulators such as BCG or other bacterial adjuvants and in some cases led to an improvement of the survival rate of patients with malignant tumours (Berd, 1990; Barth, 1994; Morton, 1992). The effect of these adjuvants is not direct, but proceeds via the excretion of a cascade of endogenous cytokines and mediators and is therefore non-specific, on the one hand, and hardly reproducible, on the other. This makes it difficult to use these non-specific adjuvants in a pharmaceutical composition.

In modern approaches, the immunostimulant cytokines are used directly as adjuvants (Rosenberg, 1988, Rosenberg, 1989). Of the immunostimulant cytokines, interleukin-2 (IL-2), interferon-α (IFN-α), interferon-γ (IFN-γ), IL-12 and GM-CSF (granulocyte macrophage stimulating factor) have proved to be particularly promising (Rosenberg, 1991; Dranoff, 1993; Zatloukal, 1993; Ferrantini, 1994; Lamont, 1996; Clary, 1996).

In trials on various cytokines it was found that there effect is strongly dependent on parameters such as 1) the site and manner of administration; 2) dosage and 3) duration of activity. Thus, for example, a systemic administration of recombinant IL-2 resulted less in the desired induction of an anti-tumoral immune response and more in serious toxic side effects and even therapy-induced deaths (Rosenstein et al., 1986; Rosenberg et al., 1989; Perez et al., 1991). The serious side effects resulted from the use of the cytokines whilst taking no account of their physiological mechanisms of activity. Cytokines are pleiotropic mediators which naturally serve to communicate between cells lying close together and operate over short distances. The release and place of activity of the cytokines are thus located close together, under physiological conditions (Pardoll, 1995). In order to obtain sufficiently high concentrations of the cytokine at the desired target site, when administering them systemically, high doses have to be given (Rosenberg et al., 1989) which lead to marked effects even at unwanted target locations.

The modern idea and the principle which a tumour vaccine sets out to achieve is to induce a systemic immune reaction directed against the tumour by means of a simultaneous, controlled delivery of appropriate tumour antigens and immunostimulatory cytokines, not by high-dose systemic administration of cytokines but rather by means of locally high doses of cytokines, acting over longer periods of time, at the site of vaccination (paracrine concept) (Pardoll, 1995; Jaffe et al., 1996).

However, the local administration of cytokines is technically problematic, particularly because the cytokines rapidly diffuse away (within minutes) into the surrounding tissues or into the blood system, coupled with an often extremely short half-life (inactivation) in biological fluids (Eppstein, 1982; Kedar et al., 1994; Koppenhagen, 1997).

In recent years it has been shown that a local release of cytokine, restricted to the vaccination site, and acting over a longer period of time can be achieved by injecting tumour cells which have been transfected with the gene coding for cytokine and have thus been made into cytokine producers (Fearon et al., 1990; Gansbacher et al., 1990; Rosenberg et al., 1992; Dranoff et al., 1993). It has been shown in animal models that vaccines based on cytokine gene-modified tumour cells are able to induce a systemic immune response directed specifically against the tumour, protecting the animals from a high-tumorigenic tumour challenge (Zatloukal et al., 1993; Zatloukal et al., 1995; Schmidt et al., 1995; Schweighoffer et al., 1996). In some cases, it was even possible to eliminate small tumours established before the vaccination (Clary et al., 1996; Clary et al., 1997). Although it is necessary to take account of the physiological paracrine mode of operation of the cytokines when adopting the approach using gene-modified tumour cells, the dosage of cytokine has proved to be another crucial parameter. Thus, it has been shown that in order to stimulate an immune response the cytokines have to be administered within a therapeutically effective dosage window; doses of cytokine which were too low were ineffective but so were excessively high doses (Zatloukal et al., 1995; Schmidt et al., 1995). On the other hand it is often difficult to achieve gene expression precisely within this effective dosage window

using the gene modification of tumour cells, especially primary tumour cells.

DE-A1 44 11 425 describes a cellular tumour vaccine which contains cytokines in delayed-release form. Specifically, it proposes IL-2 as the cytokine, taking no account of demands with regard to the dosage and release kinetics of the cytokine.

WO 94/21808 describes a tumour vaccine from autologous, cytokine gene-transfected cells, showing *inter alia* that the protective effect of cytokines (IL-2, IFN-γ and GM-CSF) is dosage-dependent, and also demonstrating that the best protective effect is not necessarily achieved by the highest dose, but not defining an optimum dosage window.

The problem of the present invention was to provide an alternative tumour vaccine which is easy to produce, which makes it possible to release the immunostimulant cytokine in controlled manner at the vaccination site in the therapeutically effective dosage range over a fairly long period.

This problem is solved according to the invention with a tumour vaccine based on tumour antigens, which is characterised in that it contains, as active ingredient, in addition to a tumour antigen source, a release system with delayed release of the active substance for IFN-γ, the effective dosage of IFN-γ being 50 ng to 5 µg and the release interval being from half an hour to 8 days.

Preferably, the IFN-γ dose is 100 ng to 2 µg, particularly 100 ng to 1 µg.

A release interval from half an hour up to 2 to 3 days has proved favourable; however, longer release intervals of up to 8 days have also demonstrated a favourable antitumour activity.

Preferably, at least about 75% of the effective IFN-γ dose is released within a release period of from one hour to 3 days.

The release of IFN-γ should start immediately if possible but at the latest one hour after administration of the vaccine. In any case, it is essential to the effectiveness of the tumour vaccine that the IFN-γ and tumour antigen source should be available substantially simultaneously.

The release system with delayed release of active substance is hereinafter referred to as the "slow release system".

Fundamentally, within the scope of the present invention, all slow release systems which satisfy the requirements regarding dosage and release of IFN-γ are suitable.

The slow release system preferably takes the form of liposomes.

Liposomes are synthetic lipid vesicles which consist of one or more concentric layers of lipid enclosing aqueous compartments. Water-soluble substances may be enclosed in the aqueous compartments whilst fat-soluble substances are incorporated in the lipid layers. Since these vesicles are suitable for a variety of uses owing to their structure, their biodegradability and low toxicity, they have increasingly been used in recent

years as carriers for all kinds of therapeutic active substances, including anti-tumour agents.

Liposomes are divided into two main categories. The first category includes the uni- or multilamellar "conventional" liposomes. Because they are rapidly absorbed by reticuloendothelial system these liposomes have a relatively short half-life.

In order to reduce non-specific interactions with cells of the reticuloendothelial system when the liposomes are used *in vivo* and in order to prevent excessively rapid breakdown, the liposomes are modified according to one embodiment of the invention. Preferably, the liposomes are modified with covalently bound polyethyleneglycol (PEG) ("PEGylated"; Mori et al., 1991; Chonn et al., 1992; Woodle et al., 1994). The quantity of PEG used is between 2 and 10% of PEG-coupled lipid in the liposome (m/m), the molecular weight of PEG is preferably between 750 and 5000 D (Klibanov et al., 1990; Blume et al., 1990; Mayhew et al., 1992; Papahadjopoulos et al., 1991; Senior et al., 1991; Mori et al., 1991; Yoshioka, 1991). The liposomes may also be modified with other groupings such as amphiphilic vinyl polymers in order to prolong their half-life *in vivo*.

The prior art provides a wide variety of liposomes and methods of producing them which are suitable within the scope of the present invention; by way of example, reference is hereby made to US Patents 4,485,045 and 4,544,545; Epstein et al., 1985; Hwang et al., 1980; EP 0 036 676; EP 0 052 322; EP 0 088 046; EP 0 102 324; EP 0 142 641; EP 0 143 949; DE 3,218,121; Eppstein, 1982; Bergers et al., 1993; Kedar et al., 1994; Herrmann and Stricker, 1995; Koppenhagen, 1997. The effect of incorporating IFN-γ in liposomes on their biological

activity has been described *inter alia* by Mellors et al., 1989 and Saravolac et al., 1996.

As an alternative to liposomes for the Slow Release System, biodegradable polymer materials in the form of microspheres may be used to incorporate IFN-γ (Maulding, 1987; Golumbek et al., 1993; Johnson et al., 1996; Lee et al., 1997; Cleland, 1997; Cleland and Jones, 1996) or Minipellets (Fujiwara et al., 1990; Marumo et al., 1997); these may also be modified to extend the half-life.

The source of tumour antigen for the vaccines according to the invention may be any compositions containing tumour antigens which are suitable for triggering a specific immune response in the individual being treated.

According to one embodiment of the invention the tumour antigens are present in the form of tumour cells.

The tumour cells of the vaccine may be autologous or allogenic tumour cells.

In one embodiment of the invention the tumour cells of the vaccines are autologous. These are cells which have been taken from the patient to be treated, optionally inactivated *ex vivo*, mixed with the slow release system which releases IFN-γ and then re-administered to the patient (methods of preparing autologous tumour vaccines are known in the art and described *inter alia* in WO 94/21808, to the contents of which reference is hereby made.).

In a preferred embodiment of the invention the tumour cells are allogenic, i.e. they do not come from the

patient who is being treated (Barth et al., 1994; Morton et al., 1992). The use of allogenic cells which are generally available in the form of established tumour cell lines is preferred particularly when economic considerations come into play; the production of individual vaccines for each individual patient is labour intensive and costly and moreover in certain patients problems arise in *ex vivo* cultivation of the tumour cells, with the result that tumour cells are not obtained in sufficiently large numbers to produce a vaccine. Preferably, a mixture of cells from a number of allogenic tumour cells lines is used as the tumour antigen source. Tumour vaccines from allogenic tumour cells are known in the art; such vaccines have been described *inter alia* by Adler et al., 1995 and Hayashi et al., 1993; Oratz et al., 1989; Morton et al., 1989; Bystryn et al., 1986.

Lysates of tumour cells such as those described by Mitchell et al., 1993 may also be used as the antigen source.

In one embodiment of the invention the tumour antigen source consists of tumour cells, particularly allogenic tumour cells, charged with peptides derived from tumour antigens. One tumour vaccine which can be used according to the invention as an antigen source in conjunction with the IFN-γ Slow Release Formulation was described by Buschle et al., 1997; as an alternative to tumour cells it is also possible to use antigen-presenting cells, e.g. dendritic cells which are charged with the tumour antigen peptides as described in DE-A1 196 07 044 or by Buschle et al., 1997.

The identification and isolation of tumour antigens and tumour-associated antigens (TAs) or peptides derived

therefrom (e.g. described by Wölfel et al., 1994 a) and 1994 b); Carrel et al., 1993; Lehmann et al., 1989; Tibbets et al., 1993; or in the published International Applications WO 92/20356, WO 94/05304, WO 94/23031, WO 95/00159), was the prerequisite for using tumour antigens as such as immunogens, as described by Anchini et al., 1996.

In one embodiment of the invention the tumour antigens are present in the form of tumour antigens as such in order to trigger a cellular immune response such as is required to eliminate tumour cells carrying tumour antigen (Bakker et al., 1994; Cox et al., 1994). The tumour antigens may be present in the form of proteins or in the form of tumour antigen-derived peptides.

One example of a cell-free tumour vaccine based on tumour antigens or peptides derived therefrom which is suitable as an antigen source within the scope of the present invention was described by Schmidt et al., 1996 and in WO 97/30721; reference is hereby made to these disclosures. A summary of cancer vaccines based on peptides which are suitable as antigen sources for the purposes of the present invention is provided by Melief et al., 1996.

The antigen source may optionally also be in the form of a slow release formulation.

A tumour vaccine based on tumour antigens, e.g. in the form of tumour cells, in conjunction with a "slow release" system in which IFN-γ is incorporated has the advantage, over tumour vaccines from gene-modified tumour cells which express IFN-γ, that the release of cytokine is precisely controlled at the vaccination site and hence the cytokine is administered in an accurate

and reproducible dosage. Moreover, the labour and hence costs involved in the manufacture are substantially reduced.

The results of the trials carried out within the scope of the present invention show that immunising mice with vaccines consisting of irradiated tumour cells and muIFN-γ-liposomes can induce a systemic immune response which protects the animals from tumour development. This protective effect demonstrates clear dependency on the dose of IFN-γ. The most effective dosage range for immunisation was found to be in the range from 100 ng to 1 µg of muIFN-γ.

It has been shown that the protective effect is dependent not only on the IFN-γ dose but also on the delayed release of the cytokine.

The delayed release of liposome-encapsulated muIFN-γ with regard to the antitumour protective effect was just as efficient as muIFN-γ expressed by genetically modified cells. Free muIFN-γ mixed in with the irradiated cells gave much reduced protection or none at all.

Both the quantity of incorporated IFN-γ and its release at the activity site are dependent on the size, shape, structure and chemical composition of the particular slow release system used.

If desired, the vaccine according to the invention may contain one or more cytokines in addition to IFN-γ, e.g. interleukin-2 (IL-2), IL-4, IL-12, IFN-α, IFN-β, IFN-ω, TNF-α. The cytokine which may additionally be contained in the vaccine may be present in the same slow release system as IFN-γ or in a different one. The cytokine may

also be made available at the site of administration by using as the source of tumour antigen tumour cells (preferably allogenic) which are transfected with the corresponding cytokine gene.

Tumour cells modified in this way and processes for preparing them are described *inter alia* in WO 95/09655, WO 95/31107, WO 93/10219, WO 93/07906 and WO 94/21808 and by Belli et al., 1997.

The vaccine may optionally also contain non-specific immunostimulant adjuvants such as LPS (lipopolysaccharide) or BCG (Bacillus Calmette Guerin).

The vaccines may conveniently be formulated as follows:

Generally, the ratio of antigen to IFN-γ dosage which is suitable for an efficient antitumour effect is determined first of all. One method of doing this is to start with a suboptimal amount of antigen for achieving an antitumour response (in the case of an optimum antigen dosage of about 10^8 tumour cells it might be, for example, about 10^7 cells) by titration, and determining the quantity of IFN-γ at which the antitumour effect increases.

Then the definitive amount of antigen is optimised.

In order to provide an effective dosage of antigen the formulation generally contains about 10^5 to 10^9, preferably 10^6 to 10^8 cells, when tumour cells are used as the antigen source; if a cell-free antigen source is used the quantity of tumour antigens or peptides derived from them is such that an immune response is triggered which is roughly equivalent to the immune response triggered by the number of tumour cells mentioned above.

If desired, another step may be carried out to check whether the addition of another cytokine or another non-specific adjuvant will bring about an increase in the antitumoral effect.

In order to select a slow release system which meets the requirements regarding the dosage of IFN-γ and the release kinetics, the following process is preferably used (the term "liposomes" is hereinafter used to indicate all preparations which permit a delayed release of protein, e.g. microspheres or minipellets):

In order to test the efficiency of charging the liposomes with IFN-γ, IFN-γ is incorporated in various liposome preparations, free IFN-γ is separated off and the IFN-γ content of the liposomes is determined, e.g. by ELISA, HPLC or using the protein measuring method according to Lowry (Lowry et al., 1951). For the biological effectiveness of the vaccines according to the invention the absolute degree of charging the liposomes with IFN-γ is not critical, but the dosage available within a certain interval of time is essential. However, it has proved advantageous if the IFN-γ concentration in the liposome preparation is at least about 10 µg/ml, preferably more than 20 µg/ml. (With a low absolute level of charging of the liposomes the desired immunomodulatory effect of IFN-γ in the vaccine can be achieved by increasing the proportion of liposome preparation in the vaccine).

In a preferred embodiment, at least 90% of the IFN-γ is enclosed in the liposomes, i.e. not more than 10% of the protein is adsorbed on the outside of the liposomes. One advantageous method of producing "alternative" liposomes of this kind consists in reducing or

preventing the non-specific electrostatic interaction which leads to the adsorption of IFN-γ, by providing a washing step using a saline solution (e.g. NaCl). The concentration of the ions must be sufficient to compete with the protein for binding to the liposomes.

In a subsequent step the release kinetics of the liposomes are determined.

In the case of liposomes the incorporation or release kinetics of the cytokines is dependent on the charge, the hydrophilic/hydrophobic nature of the cytokine, on the one hand, and on the chemical and physico-chemical characteristics of the liposomes, on the other hand. The most important characteristics of the liposomes which determine their release kinetics are their size, the number of lipid layers (uni-/multilamellar), the charge and fluidity of the lipid layers. These are in turn determined by the chemical composition of the lipid layers (Eppstein, 1982; Koppenhagen, 1997).

The principle of suitable tests for determining the release kinetics is based on incubating IFN-γ-charged liposomes in a physiological buffer system which contains serum in order to simulate conditions *in vivo* and determining the IFN-γ concentration in the supernatant at specific time intervals, e.g. by ELISA. The specialist literature relating to Slow Release Systems describes specific tests for determining the release kinetics of the therapeutic agent.

Preferably, the release kinetics are determined *in vivo*, in addition to the tests *in vitro*. Analogously to the form of administration finally adopted for therapeutic use (site of vaccination, route of administration), experimental animals are given the corresponding

injection volume which, for reasons of detectability, usually contains a larger amount of IFN-γ than the active dose of vaccine envisaged. Then blood samples are taken from the experimental animals at specified time intervals and the IFN-γ content is determined, e.g. using ELISA.

Alternatively, the *in vivo* release kinetics can be determined by administering a liposome preparation in which the liposomes and the IFN-γ contained therein have different radiolabelling. After the injection, samples are taken from the vaccination site at specified time intervals and the residual radioactivity is determined. The absolute values indicate any liposome/IFN-γ still present at the injection site; a proportional decrease in the two values leads one to conclude that the IFN-γ is still encapsulated in the liposomes.

The effective combination of tumour antigens/IFN-γ contained in the tumour vaccine according to the invention is present in such a way as to trigger a cytotoxic T-cell response and/or a humoral immune response which eliminates the tumour cells or, in the case of preventive use, ensures protection from tumour formation.

There are tests available to the skilled person for determining the extent of the immune response and for deciding on the optimum dosage of tumour antigen/IFN-γ on the basis of the test results.

The triggering of a cellular immune response can be confirmed by detecting antigen-specific CTLs (Coligan et al., 1991). Further evidence of the presence of a cellular immune response is provided if, in the absence of T-cells in an experimental animal (which is achieved

by treating the animal with antibodies which deplete CD4- or CD8-cells) no immune response takes place (Coligan et al., 1991).

A cellular immune response can also be indicated by detecting a "delayed-type hypersensitivity" (DTH) reaction in immunised animals. To do this, peptides are injected into the soles of the feet of mice and the swelling at the injection site is measured (Grohman et al., 1995; Puccetti et al., 1994). In order to measure the DTH reaction in the patient, antigens are injected intradermally and the reddening or swelling at the injection site are measured.

The induction of a humoral immune response by antigens or peptides derived therefrom which are foreign antigens to the body or antigens which are expressed in low concentrations by the body to be treated, can be determined by detecting specific antibodies in the serum. One suitable method of determining the antibody titre in the serum is enzyme linked immunoassay (ELISA). The specific antibodies are detected by a reaction with a dye after binding to the peptide used for immunisation. An alternative method is the Western blot. In this, specific serum antibodies bind to the peptide immobilised on a membrane. Bound antibodies are finally detected by reaction with a dye (Coligan et al., 1991).

In the next step, the immunomodulatory effect of the vaccines is measured in an animal trial. Established tumour models in which irradiated tumour cells induce at least a small immune response, or tumour models in which there are known tumour antigen peptide sequences recognised by immune cells, may be used for this purpose, *inter alia*. The vaccine is administered in

varying ratios of tumour antigen source to IFN-γ slow release system. The protection from tumour growth is a measurement of the effectiveness of the tumour vaccines.

The injection volume is generally 50 µl to 2 ml.

The vaccine according to the invention is generally in the form of a suspension in a pharmaceutically acceptable carrier, preferably an aqueous carrier. Aqueous carriers which may be used include for example water, buffered water, saline solution (0.4%), glycine solution (0.3%), hyaluronic acid and other known carriers. The composition may also contain pharmaceutically acceptable adjuvants such as buffer substances and inorganic salts in order to achieve normal osmotic pressure and/or effective lyophilisation. Examples of such additives include sodium- and potassium salts, e.g. chlorides and phosphates, saccharose, glucose, hydrolysed protein, dextran, polyvinylpyrrolidone or polyethyleneglycol. The compositions may be sterilised using conventional techniques, e.g. sterile filtration. The composition may be packaged directly in this form or freeze-dried and mixed with a sterile solution before use.

If desired, the tumour vaccine according to the invention may be packaged in the form of two separate formulations (tumour antigen source and IFN-γ slow release formulation) which are combined before being administered.

The tumour vaccine according to the invention may be administered prophylactically or therapeutically. It is preferably administered by intradermal, subcutaneous or intramuscular route.

Summary of the Figures

Fig. 1: Determining the release kinetics of muIFN-γ from liposomes *in vitro*

Fig. 2: Immunisation of mice with a vaccine from tumour cells and muIFN-γ (1st experiment)

Fig. 3: Immunisation of mice with a vaccine from tumour cells and muIFN-γ (2nd experiment)

Fig. 4: Determining the cytotoxic activity of T-lymphocytes after immunisation with a vaccine from tumour cells and muIFN-γ

Example 1

Incorporation of human IFN-γ in liposomes

Optimising the chemical composition and method of preparing the liposomes

Since the association of IFN-γ and liposomes is primarily charge-dependent, preliminary trials were carried out with human IFN-γ, in which the charge of the liposomes was varied. It was found that as the negative charge of the liposomes increased the efficiency of the encapsulation or association of IFN-γ improved.
For these trials, the molar ratio of the negative and neutral phospholipids (egg phosphatidylcholine (PC) and egg phosphatidylglycerol (PG)) was changed.
It was found that the association efficiency was only 20 % with a molar ratio of 1:0, whereas with a molar ratio of 1:4, 4:1 or 9:1 it rose to more than 95 %.

It was also established that the presence of salt in the hydration medium during the encapsulation reduces the efficiency of the incorporation of IFN-γ in liposomes; e.g. the rate of incorporation fell from 90 % to 35 % at a ratio of PC:PG=9:1 after the addition of 0.9 % NaCl. Consequently, instead of physiological saline solution a 5 % glucose solution was added in order to obtain an isotonic solution (this is necessary for administration).

For subcutaneous (s.c.) administration, large unsized liposomes (multilamellar vesicles) were used, because the formation of an s.c. deposit is necessary for the optimum immunostimulant activity when the vaccination is used for prophylactic or therapeutic purposes and only large liposomes remain at the injection site for a fairly long time.

The additional incorporation of cholesterol (PC:PG:cholesterol=5:1:4) in the lipid bilayers, which makes the liposomes more rigid, had no effect on either the rate of incorporation or the release kinetics of IFN-γ.

Stability tests on liposomes (PC:PG:cholesterol=5:1:4) at 4°C showed no change in stability.

The liposomes containing IFN-γ proved to be stable for at least one month at 4°C. After 30 days an IFN-γ-activity of more than >80 %, determined by HPLC, was demonstrated. The 20 % reduction in activity can clearly be attributed to a breakdown of IFN-γ; the protein measurement according to Lowry et al., 1951, showed that no appreciable amounts of protein were released from the liposomes (the storage of free IFN-γ at 4°C also shows a 20 % loss of activity).

Example 2

Induction of a systemic immune response to melanoma cells by immunisation with vaccines consisting of a mixture of irradiated tumour cells and muIFN-γ-liposomes

a) Preparation of muIFN-γ-liposomes:

Recombinant murine IFN-γ was diluted in 10 mM succinate buffer (10 mM sodium succinate, pH=5, 5% glucose) to a concentration of 100 µg/ml.

The liposomes were prepared by hydration of a lipid film as follows: Egg phosphatidylcholine (EPC, Lipoid GmbH, Ludwigshafen) and egg phosphatidylglycerol (EPG, Lipoid GmbH, Ludwigshafen) (in a molar ratio of 9:1) were dissolved in 5 parts by volume of ethanol/methanol (=solvent). (100 µmol of lipid correspond to 75 mg; 0.9 x 40 x 0.75 mg/ml of EPC and 0.1 x 40 x 0.75 mg/ml of EPG were used accordingly.) The organic solvent was evaporated *in vacuo* in a rotary flask. The thin lipid film thus obtained was dissolved with the IFN-γ solution by swirling the flask with glass beads. Using this method, mainly multilamellar liposomes (MVL) were obtained in which the IFN-γ is enclosed or onto which some of the IFN-γ may optionally be adsorbed. Any unincorporated or unadsorbed IFN-γ was separated off by ultracentrifugation at 250 000 x g for 60 min in 10 mM succinate buffer, pH=5, 10% saccharose. The liposomes contained about 86 µg of IFN-γ/ml and 36 µmol/ml of phospholipids. They were stored at 4°C.

b) Determining the release kinetics of the muIFN-γ from the liposomes *in vitro*

In order to determine the release kinetics of IFN-γ from the liposomes, liposomes containing IFN-γ were incubated in PBS/10%FCS buffer (about 0.5 - 1 ml liposomes per ml of buffer) at 37°C, which simulates an *in vivo* environment, for the time spans given in Fig.1. After incubation the samples were mixed with saccharose solution and centrifuged in order to separate the liposomes and the released protein. The lower phase was separated from the liposomes and frozen at -20°C until the IFN-γ content was determined. The content of IFN-γ was determined using an ELISA (BIO Source).

It was found that there was a delayed release of the cytokine from the liposomes. The majority of the cytokine is released within the 1st day. However, the release continues for several days and is still detectable after 8 days (Fig.1).

c) Mouse melanoma cells

The mouse melanoma cell line B16F10 (Fidler et al., 1975) comes from the NIH DCTDC Tumor Repository. The cells were cultivated in T175 culture bottles in DMEM, 10% FCS, 2 mM glutamine.

d) Preparation of the vaccines

B16F10 melanoma cells (1-2 x 10^7 cells per T175 culture bottle) were irradiated with γ-rays at a dose of 50 Gy in order to suppress further multiplication of the cells. 2-6 h after irradiation the cells were trypsinised with a trypsin/EDTA solution, washed in 3 washing steps with culture medium, PBS and Ringer's solution and adjusted to a concentration of 4 x 10^6 cells per ml (in Ringer's solution).

The cell suspension was mixed in equal parts by volume with liposome suspensions containing muIFN-γ in various concentrations (100 µg/ml, 20 µg/ml, 4 µg/ml, 0.8 µg/ml and 0 µg/ml). The finished vaccines thus contained a tumour cell concentration of 2 x 10^6 cells per ml and concentrations of liposomally encapsulated muIFN-γ corresponding to: 50 µg/ml, 10 µg/ml, 2 µg/ml, 0.4 µg/ml and 0 µg/ml.

e) Immunisation of the mice

1st experiment:

Syngenic C57Bl/6 mice (female, 8 weeks old) were immunised by subcutaneous injection of the vaccines into the rear right-hand flank. The fur was abraded on both rear flanks for this purpose. The injection volume was 100 µl per mouse. Thus 2 x 10^5 B16 cells and correspondingly 5 µg, 1 µg, 200 ng or 40 ng of liposomally encapsulated muIFN-γ were administered in each immunisation per mouse. 8 mice were immunised per dosage group.

Parallel thereto, groups of 8 mice were immunised with the following control vaccines :

- irradiated B16 cells
- irradiated B16 cells + empty(=placebo) liposomes
- irradiated B16-cells + free mu IFN-γ 5µg/ml
- mu IFN-γ genetically modified, irradiated B16-cells (the transfection was carried out with the adenovirus-aided gene transfer system based on receptor-mediated endocytosis, as described in WO 94/21808 and by Zatloukal et al., 1995; the release of mu IFN-γ was 120 ng per 24 h).

One week after the first immunisation the mice were given a booster immunisation with the same vaccines as had been used for the first immunisation.

After another week the animals were exposed to the highly tumorigenic dose of 1 x 10^5 B16 cells which was administered at a site remote from the immunisation site (on the opposite side). In addition, a group (8 animals) of non-immunised mice were exposed to the tumorigenic cells in the same way.

Eight weeks after the implanting of the tumour cells all (8/8) the unimmunised mice had developed tumours. By contrast, 4 out of 8 and 3 out of 8 animals of the groups which had been immunised with irradiated cells and muIFN-γ-liposomes, containing 200 ng or 1 μg of muIFN-γ, were protected. A similar protective effect (3/8 tumour-free animals) was found for animals which had been immunised with muIFN-γ-genetically modified cells. A merely marginal (insignificant) effect (1 out of 8 protected animals) was found for irradiated cells + liposomes containing 40 ng of mu IFN-γ-, irradiated cells + free mu IFN-γ (5 μg) and for irradiated cells + empty liposomes. Irradiated cells on their own or irradiated cells + liposomes containing 5 μg of mu IFN-γ had no protective effect. The development of the tumours in the animals is summarised in Table I and Fig. 2.

2nd experiment:

Syngenic C57Bl/6 mice (female, 9 weeks old) were immunised by subcutaneous injection of the vaccines into the right-hand hind flank. For this purpose the fur had been abraded on both hind flanks. The injection volume was 100 μl per mouse. 2 x 10^5 B16 cells and liposomally encapsulated muIFN-γ in doses of 4 μg, 1 μg, 200 ng or

40 ng were administered to each mouse per immunisation. 8 mice were immunised per dosage group.

Parallel thereto, groups of 8 mice were immunised with the following control vaccines :

- irradiated B16 cells
- irradiated B16-cells + empty (=placebo) liposomes
- irradiated B16-cells + free muIFN-γ 4 μg/ml
- irradiated B16-cells + free muIFN-γ 1 μg/ml
- irradiated B16-cells + free muIFN-γ 200 ng/ml
- muIFN-g-genetically modified, irradiated B16-cells (the release of muIFN-γ was 200 ng per 24 h).

One week after the first immunisation the mice were given a booster immunisation with the same vaccines as had been used for the first immunisation.

After another week the animals were exposed to the highly tumorigenic dose of 1 x 10^5 B16 cells which was administered at a site remote from the immunisation site (on the opposite side). In addition, a group (8 animals) of non-immunised mice were exposed to the tumorigenic cells in the same way.

Eight weeks after the implanting of the tumour cells all (8/8) of the unimmunised mice had developed tumours. By contrast, 4 out of 8 and 3 out of 8 animals of the groups which had been immunised with irradiated cells and muIFN-γ-liposomes, containing 1 μg or 200 ng of muIFN-γ, were protected. A similar protective effect (4/8 tumour-free animals) was found for animals which had been immunised with muIFN-γ-genetically modified cells. A slight protective effect (1 out of 8 animals protected) was found for irradiated cells + liposomes containing 40 ng of muIFN-γ and for irradiated cells + free muIFN-γ (1 μg). Irradiated cells or irradiated cells

+ muIFN-γ liposomes containing 4 μg of muIFN-γ as well as vaccines consisting of irradiated cells + free muIFN-γ (4 μg or 200 ng) had no protective effect. The development of the tumours in the animals is summarised in Table II and Fig.3.

Example 3

Determining the cytotoxic activity of T-lymphocytes of vaccinated animals

Syngenic C57Bl/6 mice (female, 9 weeks old) were immunised by subcutaneous injection of the vaccines into the right-hand hind flank. For this purpose the fur had been abraded on both hind flanks. The injection volume was 100 μl per mouse. 2 x 10^5 B16 cells and liposomally encapsulated muIFN-γ in doses of 3.8 μg (=high dose) and 300 ng (optimum dose) were administered to each mouse per immunisation. Parallel thereto a group was immunised only with irradiated B16 cells and another group was injected only with buffer (control group). 4 mice were immunised per group.

One week after the first immunisation the mice were given a booster immunisation with the same vaccines as had been used for the first immunisation.

After 13 days the animals were killed and their spleens were removed. The splenocytes were isolated, pooled within each group and restimulated for 5 days *in vitro* with irradiated B16 cells. On the 6^{th} day the cytotoxic activity (CTL activity) of the splenocytes (effector cells) against B16 cells (target cells) was measured using a Europium release assay (Blomberg et al., 1993). The CTL activity (expressed as % specific lysis (Zatloukal et al., 1993)) was determined for various

effector:target ratios (100:1, 50:1, 25:1) and is shown in Fig.4. Splenocytes from mice which had been immunised with irradiated B16 cells and muIFN-γ-liposomes in the optimum dosage range (300 ng of muIFN-γ) exhibited a clear cytotoxic activity against B16 cells (10 % specific lysis) compared with a very slight background activity in control animals or animals which had been immunised only with irradiated B16 cells. The increase in the muIFN-γ dose to 3.8 µg (already a high dose) led to a reduction in the CTL activity compared with the optimum dose.

Example 4

Determining the release kinetics of muIFN-γ at the vaccination site

Once it was found that muIFN-γ liposomes are an efficient adjuvant in a cellular tumour vaccine and free muIFN-γ provided no protection against a lethal tumour challenge, it was assumed that the encapsulation in liposomes results in a prolonged presence of the cytokine at the activity site. The release of muIFN-γ liposomes was therefore investigated in order to show that the liposomes stay at the injection site, the site of antigen presentation. muIFN-γ liposomes were prepared as described in Example 2a, except that the final concentration of muIFN-γ was 5 µg/ml.

In order to be able to monitor the persistence of muIFN-γ at the injection site, ^{125}I-labelled muIFN-γ was used (the specific activity corresponded to that of the ^{125}I-labelled huIFN-γ described in the following Example, likewise the mixing ratio to non-labelled muIFN-γ). In order to monitor the persistence of the liposomes at the injection site, in an alternative approach, when

preparing the lipid film, 1α,2α (n)-[^{3}H]-cholesteryl-ether, specific activity: 46 mCI (1.71 Tbq)/mMol (Amersham) was used (10 µl) in addition to PC and PG. As described in Example 2e, a single dose of 100 µl of liposomes ([^{125}I]-muIFN-γ liposomes or [^{3}H] liposomes) or free [^{125}I]-muIFN-γ (5 µg/ml) was injected into the mice subcutaneously, into the right-hand hind flank. Unlike Example 2e, no cells were added. After various lengths of time (see Fig. 5) the mice were killed. The injection site was divided into three parts. Each piece was placed in a glass scintillation test tube and 3 ml of Soluene-350 (Packard) were added in order to dissolve the samples for three days at 40°C. 500 µl of the dissolved sample were pipetted into a clean glass test tube and 250 µl of H_2O_2 were added, the test tubes being sealed once foaming had stopped. The samples were bleached for 24 h, then the addition of 250 µl of H_2O_2 was repeated twice until the samples were colourless. Finally, Hionic-Fluor (Packard) was added as the scintillation fluid and the samples were mixed in a vortex. The [^{125}I] radioactivity was measured after 24 h in a Packard multi-prias-2γ-Counter and the [^{3}H] radioactivity was measured in a Philips PW 4700 Liquid Scintillation Counter. Fig. 5 shows the percentage of the dose remaining at the injection site (ID), based on the total dose injected. It was found that the liposomally encapsulated muIFN-γ stays longer at the injection site than the free muIFN-γ.

Example 5

Determining the release kinetics of huIFN-γ at the vaccination site

a) Preparation of "conventional" huIFN-γ liposomes

The liposomes (40 μMol/ml) were prepared by the film method, substantially as described in Example 1 or Example 2, the PC and PG being mixed in a molar ratio of 9:1. For the preparation of [^{125}I]-huIFN-γ liposomes, 3.5 ml (50 μg/ml) of huIFN-γ were added to 5 % glucose (10 mM Na-succinate buffer, pH 5.0) plus 79 μl of [^{125}I]-huIFN-γ (specific activity: 13.8 μCi/ml; 10 kBq/mouse). The films were hydrated by manually shaking the flasks.

b) Preparation of "alternative" huIFN-γ liposomes

Since in preliminary trials the "conventional" liposome formulation had shown the release of a high proportion of [^{125}I]-huIFN-γ during the first hours, which can presumably be put down to the fact that this proportion immediately detaches itself from the liposomes and disappears from the vaccination site, "alternative" liposomes were prepared which contain less protein (< 10 %) adsorbed on the outer membrane.

For this purpose, lipid films consisting of PC and PG in a molar ratio of 9:1 were hydrated with a very small volume of huIFN-γ in 10 % saccharose (10 mM Na-succinate buffer, pH 5.0), e.g. 0.5 μl; 500 μg/ml. A highly viscous, gelatinous mass of highly concentrated liposomes was obtained. After 30 min this was diluted to the normal injectable concentration (40 mM of total lipid; 5 μg/ml of huIFN-γ) and washed in 0.9 % NaCl in 10 mM Na-succinate, pH 5. As the salt screens the negative charges of the lipid membrane, it prevents the electrostatic interaction with the positively charged huIFN-γ. The non-encapsulated huIFN-γ was removed by ultracentrifugation. After two washes the liposomes were taken up in 10 % saccharose buffer. The incorporation of [^{125}I]-huIFN-γ was carried out as described in a).

In order to determine the proportion of liposome-bound protein adsorbed on the outside of the liposomes, the liposomes are incubated in a trypsin solution: 20 ml (1 mg/ml) of trypsin in phosphate-buffered saline, pH 7.4, were added to 100 ml (100 mg/ml) of the liposomal dispersion (100 mg/ml of trypsin was sufficient to break down 250 mg/ml of protein). After 1 h incubation at 37°C, the enzymatic reaction was broken off during the extraction of the samples; the quantity of encapsulated huIFN-γ was determined by HPLC; it was >90 %.

c) Preparation of solution containing free huIFN-γ

The "free" [^{125}I]-huIFN-γ solution (10 kBq/mouse) was prepared by adding 52 µl of [^{125}I]-huIFN-γ stock solution (120µ Ci/ml) to 2.3 ml (50 µg/ml) of huIFN-γ in 5 % glucose (10 mM Na-succinate buffer, pH 5.0).

d) *in vivo* test

The two liposome preparations and the solution containing free huIFN-γ were injected into the mice as described in the previous Examples and the injection dose remaining was measured as described in Example 4. The results of the measurements are shown in Fig. 6, giving the percentage of the dose remaining at the injection site (ID), based on the total dose injected. It was found that the liposomally encapsulated huIFN-γ persists longer at the injection site than the free huIFN-γ, while the „alternative" liposomes exhibited even more favourable properties in terms of sustained release.

Table I

	Tumour-free mice (%) after the stated number of weeks								
vaccination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
buffer	62.5	0	0	0	0	0	0	0	0
B16	87.5	12.5	0	0	0	0	0	0	0
liposomes+B16	100	25	12.5	12.5	12.5	12.5	12.5	12.5	12.5
IFN liposomes (5 µg)+B16	100	12.5	0	0	0	0	0	0	0
IFN liposomes (1 µg)+B16	100	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5
IFN liposomes (200 ng)+B16	100	62.5	50	50	50	50	50	50	50
IFN liposomes (40 ng)+B16	100	37.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
free IFN + B16	100	37.5	25	12.5	12.5	12.5	12.5	12.5	12.5
free IFN + liposomes + B16	100	37.5	25	25	25	25	25	25	25
IFN/B16	100	62.5	50	37.5	37.5	37.5	37.5	37.5	37.5
	Number of tumour-free mice per number of injected mice after the stated number of weeks								
vaccination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
buffer	(5/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
B16	(7/8)	(1/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
liposomes+B16	(8/8)	(2/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)
IFN liposomes (5 µg)+B16	(8/8)	(1/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
IFN liposomes (1 µg)+B16	(8/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)
IFN liposomes (200 ng)+B16	(8/8)	(5/8)	(4/8)	(4/8)	(4/8)	(4/8)	(4/8)	(4/8)	(4/8)
IFN liposomes (40 ng)+B16	(8/8)	(3/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)
free IFN + B16	(8/8)	(3/8)	(2/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)
free IFN + liposomes + B16	(8/8)	(3/8)	(2/8)	(2/8)	(2/8)	(2/8)	(2/8)	(2/8)	(2/8)
IFN/B16	(8/8)	(5/8)	(4/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)

B16 = B16F10 melanoma cells irradiated with a dose of 50 Gy
IFN/B16 = B16F10 melanoma cells transfected with the gene for IFN-gamma and irradiated with 50 Gy

Table II

	Tumour-free mice (%) after the stated number of weeks								
Vaccination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Buffer	37.5	0	0	0	0	0	0	0	0
B16	87.5	37.5	0	0	0	0	0	0	0
Liposomes + B16	87.5	25	0	0	0	0	0	0	0
IFN liposomes (4 µg) + B16	100	12.5	0	0	0	0	0	0	0
IFN liposomes (1 µg) + B16	100	62.5	62.5	50	50	50	50	50	50
IFN Liposome (200 ng) + B16	100	62.5	62.5	37.5	37.5	37.5	37.5	37.5	37.5
IFN liposomes (40 ng) + B16	100	50	50	37.5	25	25	12.5	12.5	12.5
free IFN (4 µg) + B16	100	25	25	0	0	0	0	0	0
free IFN (1 µg) + B16	100	37.5	25	12.5	12.5	12.5	12.5	12.5	12.5
free IFN (200) + B16	100	0	0	0	0	0	0	0	0
IFN/B16 (200 ng/24h)	100	62.5	62.5	62.5	62.5	62.5	50	50	50
	Number of tumour-free mice per number of injected mice after the stated number of weeks								
Vaccination	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
buffer	(3/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
B16	(7/8)	(3/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
liposomes + B16	(7/8)	(2/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
IFN liposomes (4 µg) + B16	(8/8)	(1/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
IFN liposomes (1 µg) + B16	(8/8)	(5/8)	(5/8)	(4/8)	(4/8)	(4/8)	(4/8)	(4/8)	(4/8)
IFN liposomes (200 ng) + B16	(8/8)	(5/8)	(5/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)	(3/8)
IFN liposomes (40 ng) + B16	(8/8)	(4/8)	(4/8)	(3/8)	(2/8)	(2/8)	(1/8)	(1/8)	(1/8)
free IFN (4 µg) + B16	(8/8)	(2/8)	(2/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
free IFN (1 µg) + B16	(8/8)	(3/8)	(2/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)	(1/8)
free IFN (200) + B16	(8/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)	(0/8)
IFN/B16 (200 ng/24h)	(8/8)	(5/8)	(5/8)	(5/8)	(5/8)	(5/8)	(4/8)	(4/8)	(4/8)

B16 = B16F10 melanoma cells irradiated with a dose of 50 Gy

IFN/B16 = B16F10 melanoma cells transfected with the gene for IFN-gamma and irradiated with 50 Gy

Literature

Adler, A., et al., (1995), Cancer Biother 10(3): 211-224

Anchini, A., Mortarini, R., Maccalli, C., Squarcina, P., Fleischbauer, K., Mascheroni, L., and Parmiani, G. (1996), J. Immunol. 156: 208-217

Bakker, A.B.H., et al., (1994), J. Exp. Med. 179: 1005-1009

Barth, A., Hoon, D., Foshag, L., Nizze, J., Famatiga, E., Okun, E. and Morton, D. (1994), Cancer Res. 54: 3342-3345

Belli, F., et al., (1997), Cancer Immunol Immunother 44(4): 197-203

Berd, D., Maguire HC., McCue P. (1990), J. Clin. Oncol. 8: 1858-1863

Bergers, J.J., Den Otter, W., Dullens, H.F.J., Kerkvliet, C.T.M., and Crommelin, D.J.A. (1993), Pharmaceut. Research 10, 12: 1715-1721

Blomberg, K. and Ulfstedt, A.C., (1993), J. Immunol. Methods 160: 27-34

Blume et al, (1990), Biochim Biophys. Acta 1029: 91-7

Brichard, V., Van Pel, A., Wolfel, T., Wolfel, C., DePlaen, E., Lethe, B., Coolie, P., Boon, T. (1993), J. Exp. Med. 178: 489-495

Buschle, M., et al., (1997), Proc. Nat. Acad. Sci. USA 94(7): 3256-3261

Bystryn, J.C., et al., (1986), J Biol Response Mod 5(3): 211-224

Carrel, S. and Johnson, J.P., 1993, Current Opinion in Oncology 5, 383-389

Chonn, A.; Semple, S. C.; Cullis, P. R., (1992), J Biol Chem 267: 18759-65

Clary, B.M., Coveney, E., Blazer, D.G., Philip, R., and Lyerly, H.K. (1996), Surgery 120: 174-181

Clary, B.M., Coveney, Philip, R., Blazer, D.G., Morse, M., Gilboa, E., and Lyerly, H.K. (1997), Cancer Gene Therapy 4: 97-104

Cleland, J.L. and Jones, A.J., (1996),Pharm. Res. 13(10): 1464-1475

Cleland, J.L., (1997),Pharm. Biotechnol. 10:1-43

Coligan, J.E., et al., (1991), Current Prot. in Immunol., Kapitel 3, Wiley, New York

Coulie, P., Brichard, V., Van Pel, A., Wolfel, T., Schneider, J., Traversari, C., Mattei, S., De Plaen, E., Lurquin, C., Szikora, J-p., Renauld, J-c., Boon, T. (1994), J. Exp. Med. 180: 35-42

Cox, A.L., et al., (1994), Science 264: 716-719
Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D. and Mulligan, R.C. (1993), Proc. Natl. Acad. Sci. USA 90: 3539-43
Eppstein, D.A. (1982), J. Interferon Research 2, 1: 117-125
Epstein et al., (1985), Proc. Natl. Acad. Sci. (USA) 82: 3688-3692
Fearon, E.R., Pardoll, D.M., Itaya, T., Golumbek, P., Levitsky, H., Simons, J.W., Karasuyama, H., Vogelstein, B. and Frost, P. (1990), Cell 60: 397-403
Ferrantini, M., Giovarelli, M., Modesti, A., Musiani, P., Modica, A., Venditti, M., Peretti, E., Lollini, P.-L., Nanni, P., Forni, G., and Belardelli, F. (1994), J. Immunol. 153: 4604-4615
Fidler et al., (1975), Cancer Res. 35: 218-234
Fujiwara, T., Sakagami, K., Matsuoka, J., Shiozaki, S., Uchida, S., Fujioka, K., Takada, Y., Onoda, T., and Orita, K. (1990), Cancer Research 50: 7003-7007
Gansbacher, B., Zier, K., Daniels, B., Cronin, K., Bannerji, R., and Gilboa, E. (1990), J. Exp. Med. 172: 1217-1224
Gaughler , B., Van den Eynde, B., van der Bruggen, P., Romero, P., Gaforio, JJ., De Plaen, E., Lethe, B., Brasseur, F., Boon, T. (1994), J. Exp. Med.179: 921-930
Golumbek, P.T., Azhari, R., Jaffee, E.M., Levitsky, H.I., Lazenby, A., Leong, K., and Pardoll, D.M. (1993), Cancer Research 53(24): 5841-5844
Grohmann, U. et al., (1995), Eur. J. Immunol. 25, 2797-2802
Hayashi, Y., et al., (1993), Cancer 72(3): 750-759
Herrmann, J. and Stricker, H., (1995), Eur. J. Pharm. Biopharm. 41 (6): 361-368
Hwang et al., (1980), Proc. Natl. Acad. Sci. (USA) 77: 4030-4034
Jaffee, E.M., Thomas, M.C., Huang, A.Y.C., Hauda, K.M., Levitsky, H.I. and Pardoll, D.M. (1996), Journal of Immunotherapy 19 (3): 176-183
Johnson, O.F., Cleland, J.L., Lee, H.J., Charnis, M., Duenas, E., Jaworowicz, W., Shepard, D., Shahzamani, A., Jones, J.S., and Putney, SD. (1996),. Nature Medicine 2, 7: 795-799
Kedar, E., Rutkowski, Y., Braun, E., Emanuel, N. and Barenholz, Y. (1994), J. Immunother. 16: 47-59
Klibanov et al, (1990), FEBS Letters 268: 235

Koppenhagen, F.J., (1997), Liposomes as delivery system for rekombinant interleukin-2 in anticancer immunotherapy, Thesis Utrecht Universitiy.

Lamont, A.G., and Adorini, L. (1996). IL-12: a key cytokine in immune regulation. Immunol Today 5: 214-217

Lee, H.J., et al., (1997), J. Pharmacol. Exp. Ther. 281(3): 1431-1439

Lehmann, J.M., et al., (1989), Proc. Natl. Acad. Sci. USA 86, 9891-9895

Longo, W.E. and Goldberg, E.P., (1985), Methods Enzymol. 112: 18-26

Lowry, O.H., et al., (1951), J. Biol. Chem. 193: 265-275

Marumo, K., et al., (1997), Int. J. Urol. 4(1): 55-61

Maulding, H.V., (1987), J. of Controlled Release 6: 167-176

Mayhew et al, (1992), Int. J. Cancer 51: 1-8

Melief, C. JM. et al., (1996), Current Opinion in Immunology (8), 651 - 657

Mellors, J.W., et al., (1989), Infect. Immun. 57(1): 132-137

Mitchell, M.S., et al., (1993), Ann. NY Acad. Sci. 12; 690: 153-166

Mori, A.; Klibanov, A. L.; Torchilin, V. P.; Huang, L., (1991), FEBS Lett 284: 263-6

Morimoto, Y. and Fujimoto, S., (1985), Crit. Rev. Ther. Drug Carrier Syst. 2(1):19-36

Morton, D.L., et al., (1989), Semin Surg Oncol 5(6): 420-425

Morton, D., Foshag L., Hoon D., Nizze J., Famatiga E.m Wanek L., Chang C., Davtyan D., Gupta R. and Elashoff R. (1992), Ann Surg 216: 463-482

Oratz, R., et al., (1989), J Biol Response Mod 8(4): 355-358

Papahadjopoulos et al, (1991), Proc. Natl. Acad. Sci. USA 88: 11460-4

Pardoll, D.M. (1995), Ann. Rev. Immunol. 13: 399-415.

Perez, E.A., Scudder, S.A., Meyers, F.A., Tanaka, M.S., Paradise, C. and Gandara, D.R. (1991), J. Immunother. 10: 57-62

Puccetti, P. et al., (1994), Eur. J. Immunol. 24, 1446-1452

Rosenberg, SA. (1988). Immunotherapy of patients with advanced cancer using interleukin-2 alone or in combination with lymphokine activated killer cells. In Important Advances in Oncology, ed. V de Vita, S Hellman, SA Rosenberg, pp. 217-257.Philadelphia: JB Lippincott.

Rosenberg, SA., Lotze, MT., Yang, JC., Aebersold, PA., Lineham, WM., Seipp, CA., Withe, DE. (1989), Ann. Surg. 210: 474-485

Rosenberg, S.A. (1991), Cancer Res. 51: 5074-5079.

Rosenberg, S.A., Anderson, W.F., Blaese, M.R., Ettinghausen, S.E., Hwu, P. et al. (1992), Human Gene Therapy 3: 75-91

Rosenstein, M., Ettighausen, S.E. and Rosenberg, S.A. (1986), J. Immunol. 137: 1735-1742

Saravolac, E.G., et al., (1996), Antiviral Res. 29(2-3): 199-207

Schmidt, W., Schweighoffer, T., Herbst, E., Maass, G., Berger, M., Schilcher, F., Schaffner, G. and Birnstiel, M.L. (1995), Proc. Natl. Acad. Sci. USA 92: 4711-4714

Schmidt, W., et al., (1996), Proc. Natl. Acad. Sci. USA 93(18): 9759-9763

Schmidt, W., et al., (1997), Proc. Natl. Acad. Sci. USA 94(7): 3262-3267

Schweighoffer, T., Berger, M., Buschle, M., Schmidt, W., and Birnstiel, M.L. (1996), Cytokines Mol. Ther. 2: 185-192

Senior, J.; Delgado, C.; Fisher, D.; Tilcock, C.; Gregoriadis, G., (1991), Biochim Biophys Acta 1062: 77-82

Tibbets, L.M., et al., (1993), Cancer, Jan. 15., Vol.71, 2, 315-321

Torchilin, V. P.; Klibanov, A. L.; Huang, L.; S, O. D.; Nossiff, N. D.; Khaw, B. A., (1992), Faseb J 6: 2716-9

Torchilin, V. P., et al., (1994), Biochim Biophys Acta 1195: 181-184

Torchilin, V. P., and Papisov, M. I., (1994), J Liposome Res 4(1): 725-739

van der Bruggen, PC., Traversari, C., Chomez, P., Lurquin, C., DePlaen, E., Van den Eynde, B., Knuth, A., Boon, T. (1991), Science 264, 1643-1650

Willmott, N., et al., (1989), J. Pharm. Pharmacol. 41(7): 433-438

Woodle, M. C.; Newman, M. S.; Cohen, J. A.,(1994), J Drug Target 2: 397-403

Wölfel, T. et al., (1994) a), Int. J. Cancer 57, 413-418

Wölfel, T. et al., (1994) b), Eur. J. Immunol. 24, 759-764

Yoshioka, (1991), Biomaterials 12: 861-4

Zatloukal, K., Schmidt, W., Cotten, M., Wagner, E., Stingl, G. and Birnstiel, M.L. (1993), Gene 135: 199-207

Zatloukal, K., Schneeberger, A., Berger, M., Schmidt, W., Kosik, F., Kutil, R., Cotten, M., Wagner, E., Buschle, M., Maass, G., Payer, E., Stingl, G. and Birnstiel, M.L. (1995), J.Immunol. 154, 3406-3419

Zier, K. and B. Gansbacher. (1995), Hum. Gene Ther. 6:1259-1265

Patent Claims

1. Tumour vaccine based on tumour antigens, characterised in that it contains, as active constituent, in addition to a tumour antigen source, a release system with delayed release of the active substance for IFN-γ, the effective dose of IFN-γ being 50 ng to 5 μg and the release interval being from half an hour to 8 days.

2. Tumour vaccine according to claim 1, characterised in that the effective dose of IFN-γ is 100 ng to 2 μg.

3. Tumour vaccine according to claim 2, characterised in that the effective dose of IFN-γ is 100 ng to 1 μg.

4. Tumour vaccine according to one of claims 1 to 3, characterised in that the release interval is from half an hour to 2 to 3 days.

5. Tumour vaccine according to claim 4, characterised in that about 75% of the dose of IFN-γ is released within an interval of between one hour and 3 days.

6. Tumour vaccine according to one of claims 1 to 5, characterised in that the release system with delayed release of the active substance consists of liposomes.

7. Tumour vaccine according to claim 6, characterised in that the liposomes contain >90 % of the IFN-γ enclosed therein and <10 % adsorbed on the outside.

8. Tumour vaccine according to one of claims 1 to 5, characterised in that the release system with delayed release of the active substance consists of microspheres.

9. Tumour vaccine according to one of claims 1 to 5, characterised in that the release system with

delayed release of the active substance consists of minipellets.

10. Tumour vaccine according to one of claims 1 to 9, characterised in that the tumour antigen source consists of tumour cells.

11. Tumour vaccine according to claim 10, characterised in that the tumour cells are allogenic tumour cells.

12. Tumour vaccine according to claim 10 oder 11, characterised in that the tumour cells are charged with peptides derived from tumour antigens.

13. Tumour vaccine according to one of claims 1 to 9, characterised in that the tumour antigen source consists of antigen-presenting cells which are charged with tumour antigen peptides.

14. Tumour vaccine according to one of claims 1 to 9, characterised in that the tumour antigen source consists of tumour antigens as such or peptides derived therefrom.

Abstract

A tumour vaccine based on tumour antigens contains as active constituent, in addition to a tumour antigen source, a release system with delayed release of active substance for IFN-γ, the effective dose of IFN-γ being 50 ng to 5 μg which is released over a period of from several hours to several days. The release system for IFN-γ preferably consists of liposomes, while the tumour antigen source preferably consists of allogenic tumour cells.

Fig. 1/6
Fig. 1

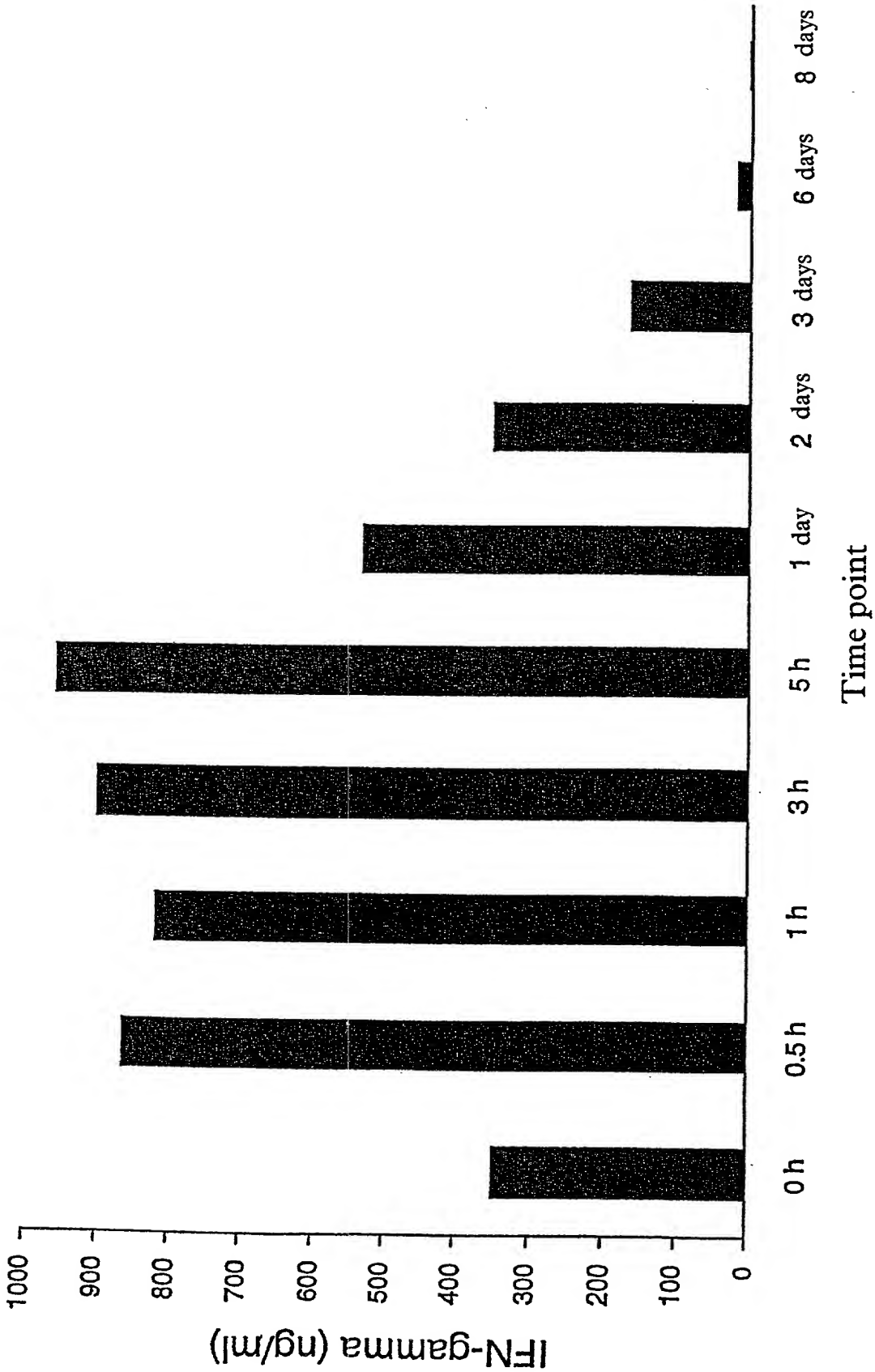

IFN-gamma (ng/ml)
1000
900
800
700
600
500
400
300
200
100
0
0 h
0.5 h
1 h
3 h
5 h
1 day
2 days
3 days
6 days
8 days
Time point

Fig. 2/6

Fig. 2

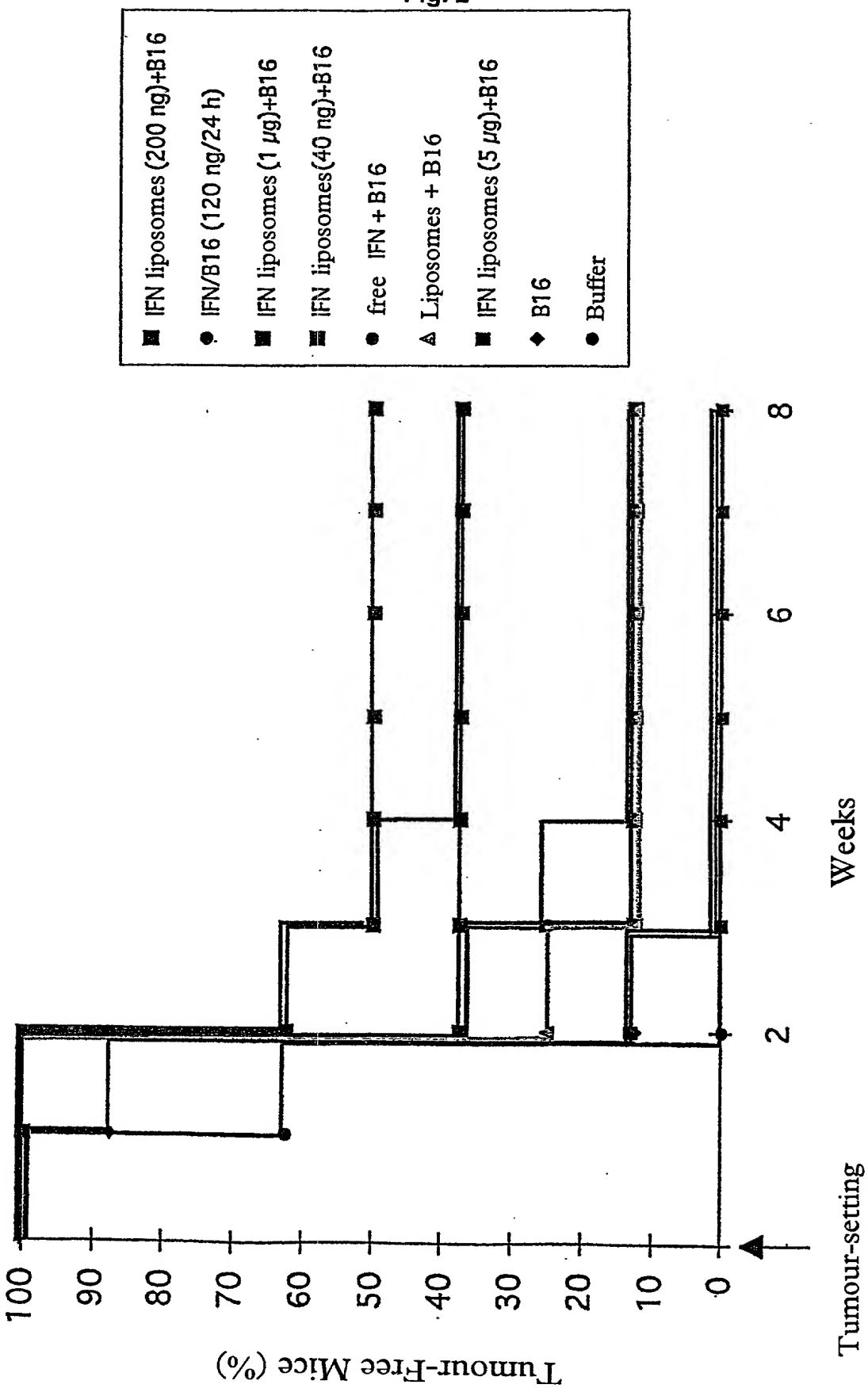

IFN liposomes (200 ng)+B16
IFN/B16 (120 ng/24 h)
IFN liposomes (1 µg)+B16
IFN liposomes(40 ng)+B16
free IFN + B16
Liposomes + B16
IFN liposomes (5 µg)+B16
B16
Buffer
Tumour-Free Mice (%)
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
Weeks
Tumour-setting

Fig. 3/6
Fig. 3

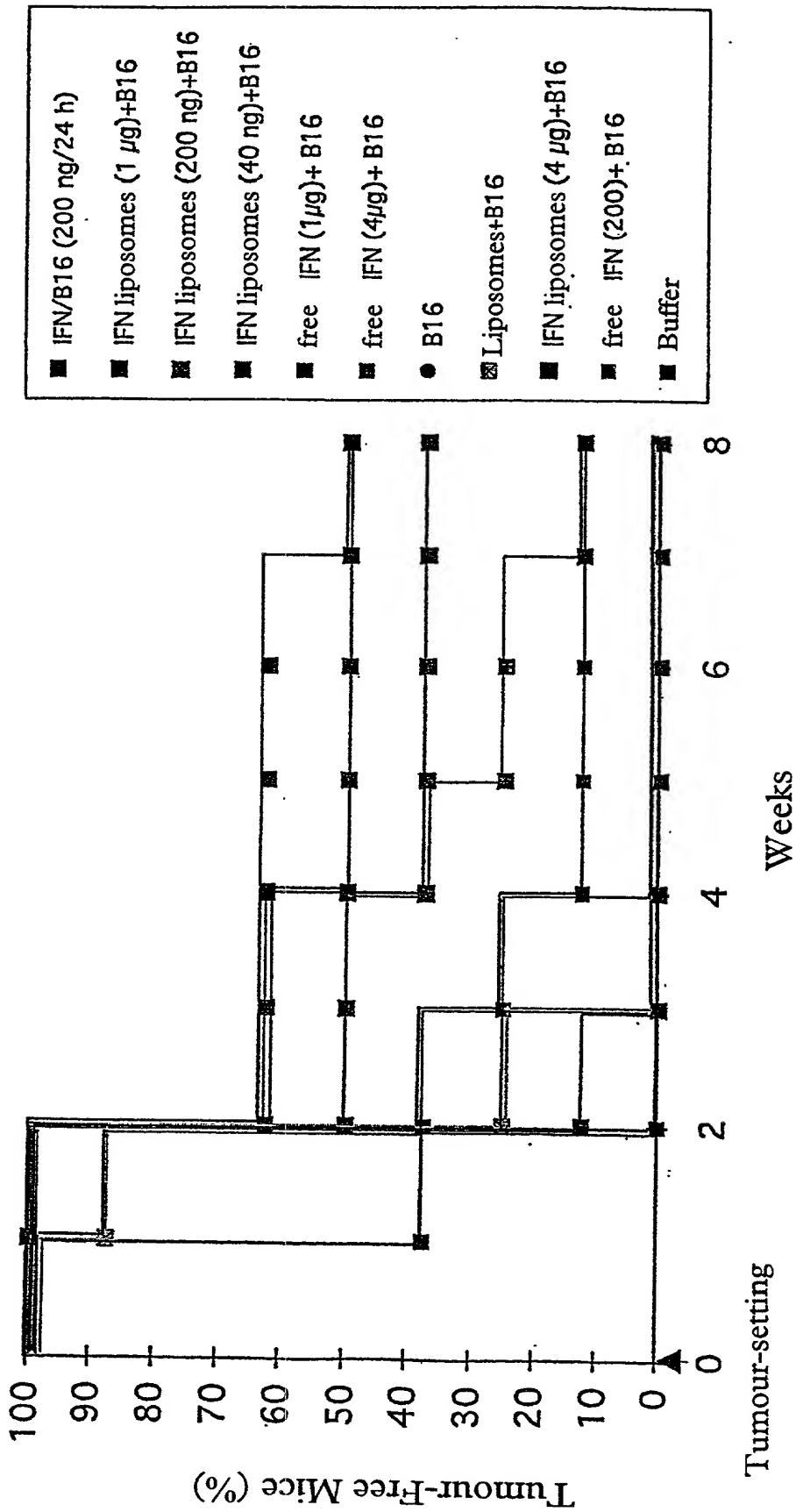

IFN/B16 (200 ng/24 h)
IFN liposomes (1 µg)+B16
IFN liposomes (200 ng)+B16
IFN liposomes (40 ng)+B16
free IFN (1µg)+ B16
free IFN (4µg)+ B16
B16
Liposomes+B16
IFN liposomes (4 µg)+B16
free IFN (200)+ B16
Buffer
Tumour-Free Mice (%)
100
90
80
70
60
50
40
30
20
10
0
Tumour-setting
0
2
4
6
8
Weeks

Fig. 4/6
Fig. 4

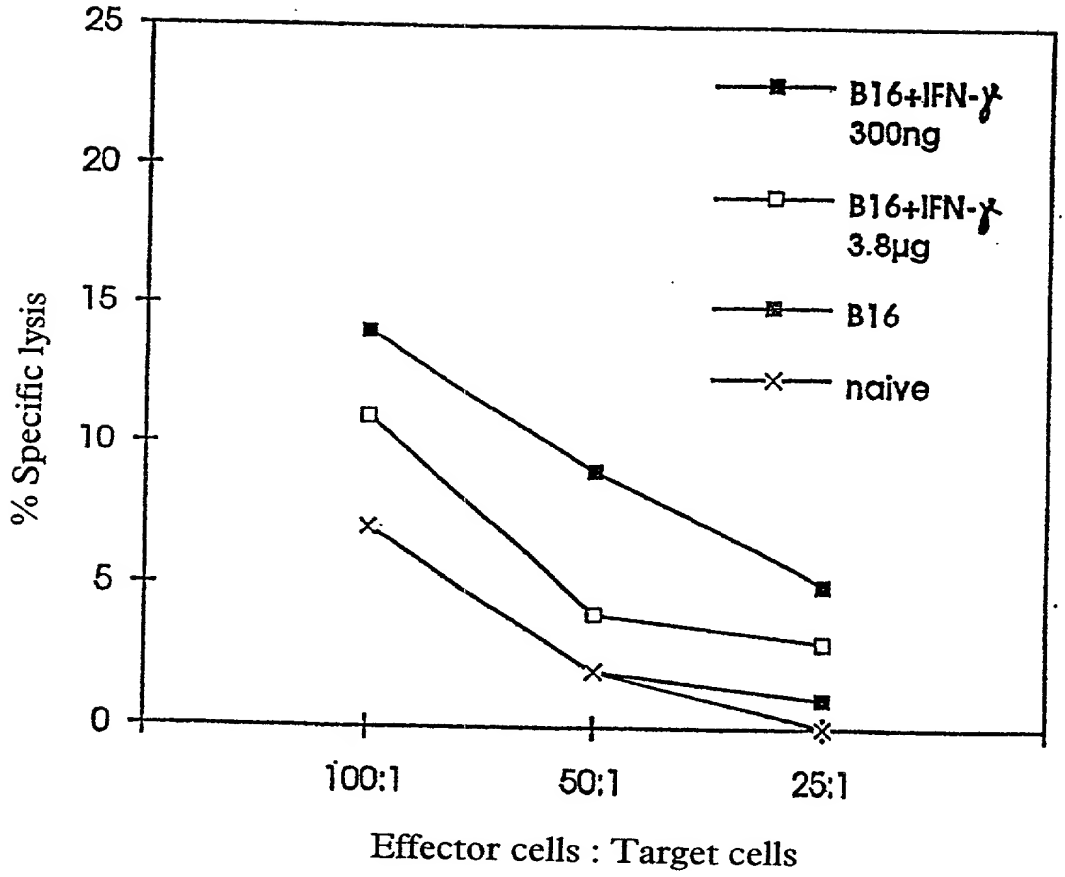

25
20
15
10
5
0
% Specific lysis
B16+IFN-γ 300ng
B16+IFN-γ 3.8µg
B16
naive
100:1
50:1
25:1
Effector cells : Target cells

Fig. 5/6
Fig. 5

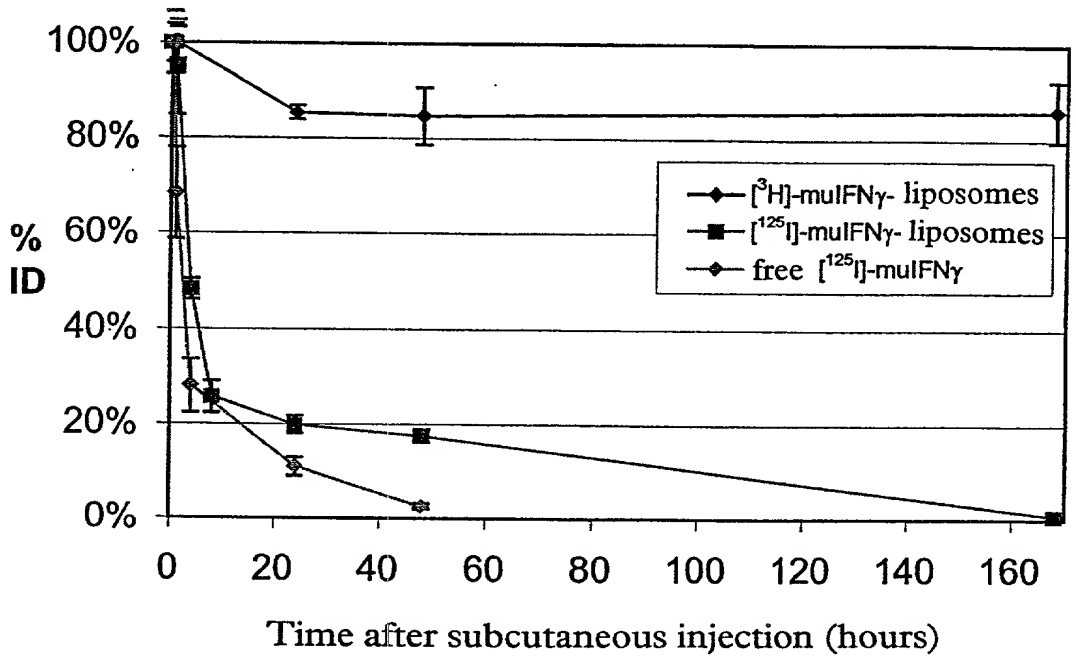
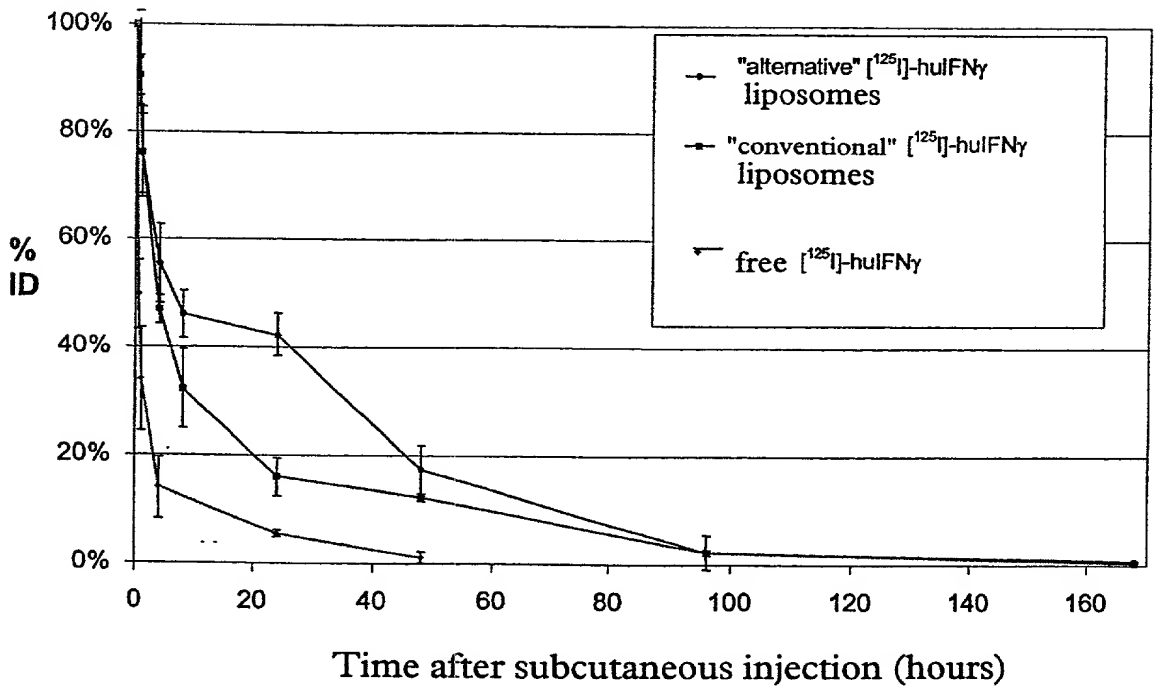

100%
80%
60%
40%
20%
0%
% ID
0
20
40
60
80
100
120
140
160
[3H]-mulFNγ- liposomes
[125I]-mulFNγ- liposomes
free [125I]-mulFNγ
Time after subcutaneous injection (hours)

Fig. 6/6
Fig. 6


100%
80%
60%
40%
20%
0%
% ID
0
20
40
60
80
100
120
140
160
"alternative" [125I]-hulFNγ liposomes
"conventional" [125I]-hulFNγ liposomes
free [125I]-hulFNγ
Time after subcutaneous injection (hours)

12 A83

Declaration for Patent Application

Docket Number: 0652.2050000

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled Tumour Vaccine, the specification of which is attached hereto unless the following box is checked:

☒ was filed on 15 October 1998;
as United States Application Number or PCT International Application Number PCT/EP98/06546; and
was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application, which designated at least one country other than the United States listed below, and have also identified below any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Claimed	
DE 197 46 173.5	Germany	18 October 1997	☒ Yes	☐ No
(Application No.)	(Country)	(Day/Month/Year Filed)		
_____	_____	_____	☐ Yes	☐ No
(Application No.)	(Country)	(Day/Month/Year Filed)		

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

_____	_____
(Application No.)	(Filing Date)
_____	_____
(Application No.)	(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56 that became available between the filing date of the prior application and the national or PCT international filing date of this application.

PCT/EP98/06546	October 15, 1998	Pending
(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)
_____	_____	_____
(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)

Send Correspondence to:

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.
1100 New York Avenue, N.W.
Suite 600
Washington, D.C. 20005-3934

Direct Telephone Calls to:

(202) 371-2600

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Ernst WAGNER	
Signature of sole or first inventor	Date 19 April 2000
Residence Langenzersdorf, Austria ATX	
Citizenship AUSTRIA	
Post Office Address Wienerstraße 201, A-2103 Langenzersdorf, Austria	

Full name of second inventor Ralf KIRCHEIS	
Signature of second inventor	Date 18 April 2000
Residence Vienna, Austria ATX	
Citizenship GERMANY	
Post Office Address Ziedlergasse 16/3/5, A-1230, Vienna, Austria	

Full name of third inventor Daan J.A. CROMMELIN	
Signature of third inventor	Date 20 May 2000
Residence Utrecht, Netherlands NLX	
Citizenship NETHERLANDS	
Post Office Address Frederik Hendrikstraat 98, NL - 3500 NA, Utrecht, Netherlands	

Full name of fourth inventor Maaike van SLOOTEN	
Signature of fourth inventor	Date 28 mei 2002
Residence Utrecht, Netherlands NLX	
Citizenship NETHERLANDS	
Post Office Address Havikstraat 17, NL - 3500 NA, Utrecht, Netherlands	
Full name of fifth inventor Gert STORM	
Signature of fifth inventor	Date 31 May 2002
Residence Amersfoort, Netherlands NLX	
Citizenship NETHERLANDS	
Post Office Address Elkerlijkpad 12, 3813 CN Amersfoort, Netherlands	

P:\USERS\BRIAND\0652\2050000\Declaration
SKGF Rev 9/2/98 mac

(Supply similar information and signature for subsequent joint inventors, if any)